CONTROLLED VASCULAR CELLULAR GROWTH BY MICROSTTRUCTURE PATTERNED SURFACES ON IMPLANTABLE BIODEGRADABLE POLYMER SCAFFOLD

by

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ABSTRACT

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The technological advances in the field of MEMS and biodegradable polymers with elastomeric properties have opened new areas of application for engineering of soft tissues such as blood vessel, heart valves, cartilage, tendon, and bladder, which exhibit elastic properties. In this thesis work, Microfabrication technology has successfully been applied to biodegradable polymer films to encourage cell growth in designated area. This work is presented with the final goal of fabricating implantable polymer scaffolds to produce small diameter blood vessels in a controlled manner spatially by using the concept of wettability and contact guidance.

There are several methods to control cell growth including patterned chemical cues on the surface. Although successful in patterned cell growth, chemical cues are not suitable for implantation. While the concept of “contact guidance” uses physical cues to guide cell growth most of the research has been done on either conventional microelectronic materials such as silicon and metals or non-implantable polymers such as PDMS.

Thus, we have introduced microscale roughness to the CUPE biodegradable polymer film surface by making microdome structures and have achieved a very high contact angle on

polymer surface, where cell adhesion is preferentially much lower than on smooth scaffold surfaces.

We have also successfully transferred microchannel structure patterns over the CUPE polymer, and NIH 3T3 cell growth patterns were observed for a 5 day study. The results of the patterned cell study were compared with the cells cultured on polymer films without micropattern. The results indicated that cells exhibited aligned and elongated morphology in microchannels. The cells on film without micropatterns were randomly oriented.

This *in-vitro* study of microfabricated films proves the concept of selective and guided cell growth without using any chemical modification on implantable scaffold.

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CHAPTER 1

INTRODUCTION

1.1 Tissue engineering

Tissue engineering is an emerging interdisciplinary research area, which has the potential to regenerate or replace damaged tissues with laboratory-grown parts such as bone, cartilage, blood vessels and skin. Although In today's world, organ transplantation biology and techniques have advanced exponentially over the past decades, limited donor organ supply and organ rejection have always been a cause for concern. A large number of patients suffer from the loss, damage or failure of a single organ or tissue component (Bhatia and Chen, 1999). Tissue engineering technology can be the most promising way to handle this problematic situation. Despite being in its early stages of development, the pace of research and the growth in this field has been phenomenal, showing promising progress in the field. Tissue engineering is a novel field and works with a very simple concept: It involves the use of a biomaterial construct combined with tissue specific cells either by combining the two components prior to implantation or by encouraging cells to populate the construct after implantation in a host [1, 2, 3]. A wide variety of biomaterials with a number of different structures have been used to develop tissue constructs. Till today, four types of biomaterials have been studied as scaffold materials: Synthetic organic materials, synthetic inorganic material, organic materials of natural origin collagen, and inorganic material of natural origin. Examples are ceramics, polyurethanes or collagen and coralline hydroxyapatite respectively. Hollister et al. stated that the purpose of scaffolds is to provide three dimensional space to facilitate cell growth by providing a favorable microenvironment so that it can resemble the final tissue construct [6]. In general, scaffold is fabricated from a highly biocompatible material, which does not create immune response in body and also its material is chosen such that it can degrade and resorb at a controlled rate.

Although progress is continuously being made to understand and generate the universal tissue scaffold, our knowledge about the biology of tissue formation is still empirical and not well understood. It is well known that, besides surface chemistry of the scaffold material, the fiber diameter, pore size, porosity and microstructure can regulate tissue development. In spite of this, how these parameters plays role are not clear so far. Despite of this, tissue engineering products are already available for clinical use. Regeneration of skin, bone, and blood vessels using delivery of recombinant growth factor will be possible in the very near future [4, 5]. The overall objective of this research is to study the cell growth and behavior on various patterned microstructured surfaces to find out the reason for unanswered questions and use behavioral response for application purposes in three dimensional tissue scaffolds for animal cell growth in desired areas. Tissue engineering systems precisely define the microenvironment developed by cell type, extracellular matrix, cell-cell interaction, growth factors and many others. Since this work mainly focuses on the study of animal cell culture in three dimensions for the purpose of tissue engineering, associated topics will be discussed in the following section.

Animal cell culture is the process to continuously grow animal cells *in vitro* after removing them from animal tissue. This cell culture technique has paved the way in the field of biology, medicine and tissue engineering. It gives researches a chance to study about cell behavior, interactions, the role of growth factor and provides a platform to investigate the normal physiology and biochemistry of cells. Other than scientific study, it also opened up a plethora of windows for applications like- *in vitro* drug testing, production of therapeutically significant biological compounds like hormones and proteins, widely used vaccines and the production of artificial tissue for implantation. Primary cell culture is done after disaggregating cells either mechanically or enzymatically from desired piece of tissue. Most normal cells are anchorage dependent. These cell lines have been extensively used in tissue engineering filed for research and application purposes. All cells in tissues are in contact with each other through a complex network of extracellular proteins known as the extracellular matrix (ECM). This matrix usually

provides structural support to the cells, and also provides a medium for the cells to interact and migrate [7]. The ECM is composed of an interlocking mesh of glycosaminoglycans (GAGs) and fibrous protein, mainly secreted by cells. There are two functional types of fibrous proteins: the mainly structural ones, like collagen and elastin, and the mainly adhesive ones, like fibronectin and laminin. Also, cells may have different shapes when attached to the two dimensional surface or in a three dimensional space with cell-cell interactions. Cell-extra cellular matrix (ECM) interaction is important for adhesion, migration, proliferation and differentiation in cells. Cell-ECM junction can be observed in cell culture as focal adhesion. Without adhesion most cells initiate apoptosis, which leads to cell death. Similar to Cell-ECM interaction, Cell-Cell interactions also play a very important role in tissue culture. It is essential for normal organ development and functionality.

1.2 Factors Affecting Cell Attachment to Scaffolds

Cell attachment to the surface is complex phenomena and various factors play crucial roles in determining the cell response to the scaffold. Initial response of cells to the biomaterial mostly depends on the surface properties. Therefore surface modification either to its surface chemistry or surface topography has been investigated by researchers to learn cell behaviors. Surface modification technologies have been recently used to control cell behavior for application purposes. Therefore it is important to understand the factors affecting cell attachment and behavior. To obtain a tissue construct, one has to start by seeding cells onto a scaffold and then provide optimum growth conditions to allow cell proliferation and growth.

1.2.1 Surface Chemistry

Fibroblast spreading on any surface depends on the surface free energy. Surface chemistry also affects cell attachment and spreading [8] and specific chemical groups at the polymer surface like hydroxyl [9] or surface –C-O functionalities have been identified as important factors in modulating the fate of cell attachment. Density of surface –OH groups [9], surface sulfonic groups [10], and surface free energy [11] has been identified as parameters that can modulate

cell attachment. Many polymers have been widely used as a polymer substrate for cell growth by changing surface chemistry. The ability of polymers to adsorb extra cellular matrix proteins like fibronectin onto their surface has been identified as a major factor which mediates cell attachment to polymer surfaces [12]. In fact, surface chemistry affects the degree of ECM protein adsorption which in turn can regulate cell differentiation and proliferation. There are receptor proteins on the surface of the CHO cells called integrins which can bind with fibronectin and proliferate on the surface. Integrins are transmembrane proteins which are linked to the cytoskeleton and thereby anchor the cells to the ECM. Once several cells have attached to the surface via the integrin-fibronectin interaction, the cells will start spreading out and growing. When the cell culture becomes confluent, cells can attach to each other via proteins called the cadherins.

1.2.2 Nano and Micro Scale structure

Cell morphology, proliferation, differentiation and apoptosis depend on the scaffold structure. While the chemistry of the ECM and the presence of specific soluble cytokines and metabolic factors are usually needed to modulate cellular response, microfabrication tools have recently aided in the identification of previously ignored parameters of the cellular microenvironment [3]. For example, Chen et al. showed that adhesion to the ECM regulates capillary endothelial cells to proliferate or undergo apoptosis by changing cell shape [28]. Progressively restricting bovine and human endothelial cell extension by culturing cells on smaller and smaller micropatterned adhesive islands regulated a transition from growth to apoptosis [13]. The study also showed the relation between cell morphology and function as apoptotic cells had increased spherical morphology as compared to spreading cells. However, it is interesting to note that the cells when forced to adopt a spherical morphology, could not spread because the neighboring micropatterned island with adhesive coating was too far away for the cell to bridge across the non-adhesive portion. They report that, when the islands were closer, the cells were not apoptotic as they could spread from one island to another by bridging the gap between them.

This hints at the fact that cell size also dictates the physical surroundings it prefers for growth. Craighead et al., reported that astrocytes grew on the tops of columnar arrays (0.5 μm dia, 1 μm apart and 1 μm tall),However, when the column features were replaced with a surface with nanometer scale features (~ 60 nm in diameter and ~ 230 nm tall) the cells did not spread well [14]. Apart from two dimensional, micropatterning, the macro and micro structure of the tissue engineering scaffolds and their surface chemistry have shown regulatory effects on cell growth, morphology, proliferation, differentiation and apoptosis [15, 16, 17]. Although the macroscale organization of microfabricated scaffolds is totally different from foamed scaffolds, which in turn has different organization as compared to non woven meshes, pore size does matter when it comes to cell morphology [18].

Although no general principles have emerged which can predict the extent of attachment of cells to polymer surfaces, Surface chemistry, cell seeding, Nano and micro Scale structures all collectively play role in cell attachment to scaffolds.

1.3 Micro-channels for small diameter blood vessels

Disorders of the vascular system results in significant morbidity, mortality and accounts for a big segment of health care expenditures. Coronary artery atherosclerotic disease and peripheral vascular disease are the most frequent causes of death in the United States. Abnormalities in the endothelium's ability to regulate permeability to macromolecules contribute to the development of atherosclerosis, the arterial disease whose pathological, namely heart attack and strokes, are leading cause of mortality [19].

Surgical intervention in the form of vascular bypass grafting is commonly required. Although autologous vessels remain the standard for small diameter grafts, many patients do not have a vessel suitable for use because of vascular disease, amputation, or previous harvest. As a result, tissue engineering has emerged as a promising approach to address the shortcomings of current therapies.

Although tissue engineering of blood vessels has been under way for more than two decades and has seen important advances, [20-21] poor mechanical strength and vasoactivity plague (atherosclerosis) have remain unsolved problems for tissue engineered blood vessels(TEBVs).

It has been shown that early atherosclerosis lesions develop preferentially in the arterial region within which endothelial cells (ECs) are round (or cubodial), while arterial regions largely resistant to development of atherosclerosis are characterized by elongated ECs [22]. This suggest that the extend of EC elongation may be an important regulator of ECs physiology. Control of EC elongation has previously been demonstrated using a number of different strategies. For instance, exposure of cultured ECs for several hours to a steady fluid mechanical shear stress greater than approximately 7 dyne/cm2 leads to extensive cytoskeleton reorganization that results in progressive cellular elongation and alignment in the direction of flow [22, 23, 24] However, shear stress is relatively invasive in that it also elicits a wide spectrum of EC metabolic and gene regulatory responses that alter cell phenotype [25 26]. Thus, it is impossible to determine whether functional differences are due to differences in cell shape or result of flow-induced phenotypic changes.

Control of cell shape without flow has been demonstrated in vitro by culturing cells on varying the density of extracellular matrix proteins on which cells are cultured [27], and by patterning surfaces with self-assembled monolayers to which cells preferentially adhere [28 29 30]. However, adsorption of proteins present in cell culture media to patterned and modified surfaces often limits the lifetime and usability of these surfaces. Therefore, the need remains for a relatively non-invasive capability to control EC shape while simultaneously permitting cell imaging.

Biodegradable scaffolds with 3D microchannels separated by high aspect ratio microwalls are useful for elongation of cell and emulating the microenvironment. However, there are no reports of the fabrication of microchannels separated by high aspect ratio microwalls on biodegradable

polymer with EC culture. High aspect ratio microwalls are difficult to demold because they tend to break in the micromold.

Studies have been done with microchannel structures to align and elongtae the cells but none of those have resulted in an appropriate solution. Most of studies on are limited to Smooth muscle cells. Sarkar et al showed that grooves (20–80 mm wide and 5 mm deep) in nonbiodegradable polydimethylsiloxane (PDMS) can control the vascular SMC aspect ratio, alignment, and oriented remodeling of the ECM [31]. However, nondegradable scaffolds affect the vasoactivity of the remodeled vessel. Shen et al. showed a liquid UV polymerizable biodegradable macromer PCLLGA diacrylate was synthesized and used for ultraviolet (UV) microembossing [32]. Micropatterned films with microchannels (depth 60–70 μm , widths10 μm to 160 μm) separated with narrow microwalls (10 or 25 μm) were made. The effect of the microstructures on cell growth, orientation, and phenotype control of vascular SMCs was studied but biodegradability and mechanical strength of polymer was still an issue.

Gray et al. have described a microfabricated platform for inducing varying degrees of EC elongation. The platform consists of microchannels of different widths within which ECs were cultured. The results showed that the shape index, a dimensionless measure of cell roundness, decreases from 0.75±0.01 (mean±SEM) in 225μm-wide microchannels to 0.31±0.02 in 25μm-wide channels [19]. However, this study was perfomed on collagen coated silicon microchannels, still stating the need to develop biodegradable scaffolds with 3D microchannels structures for implantation.

To address aforementioned issues, in this research we have successfully shown the fabrication of microchannels on biodegradable and mechanically strong polymer by soft lithography techniques. Microchannel pattern was transferred over the CUPE polymer and 20μm deep and 20μm wide channels were obtained. We have shown the cell alignment results with NIH 3T3 fibroblast cells on microchannel CUPE scaffold, with future improvement of endothelial cell culture and complete scaffold formation.

1.4 Objective of research project

1.4.1 Specific Aims

The ultimate goal of this project is to create the three dimensional scaffold by aligning the cells in one directions to resemble the *in-vivo* small diameter blood vessel. To achieve this goal followings aims were set for this project:

1. Fabrication of various microstructures on silicon mold with different dimensions.
2. To transfer the pattern from mold to biomaterial scaffold to provide alignment to the cells.
3. To study the effect of surface topography on cell growth, morphology and alignment.

1.4.2 Impact of Project

The successful results of this project will impact the cardiovascular tissue engineering approach by providing cell patterning and alignment just by applying micofabrication technologies to tissue scaffolds. The prospective outcome of the project will open up the possibility to fabricate biodegradable small diameter blood vessels for treatment of cardiovascular diseases.

1.4.3 Thesis outline

The Oncoming chapters of the thesis are discussed as follows.

Chapter 2 describes about MEMS, its principle and application of MEMS in tissue engineering field.

Chapter 3 explains about polymer and its property for scaffold making.

Chapter 4 explains the design and experimental setup from mold fabrication to cell culturing.

Chapter 5 describes the results and discussion of this project.

Chapter 6 briefly mention about the microfluidic device fabrication and real time cell culture

Chapter 7 discusses the future study with conclusion of the current results.

CHAPTER 2

MEMS AND BIO-MEMS

Micro-Electro-Mechanical Systems (MEMS) as the name suggests, is the integration of mechanical elements, sensors, actuators, and electronics on a common silicon substrate through microfabrication technology. This two and half decade old technology has revolutionized the electronics industry by bringing together silicon-based microelectronics with micromachining technology. Usually MEMS are made up of components between 1 to 100 micrometres in size by using microengineering tools. Microengineering refers to the technologies of making three dimensional structures and devices with micrometers of dimensions. MEMS have attained the researcher's interest for biology or tissue related research because this technology deals with micron range features comparable to tissue or cell size. As research into understanding of basic mechanism of life expands down to single-cell and molecular level, the need for more complex cell culture studies arises. This leads to the use of MEMS technology for biology application, coined as Bio-MEMS. Tissue scaffold related research has been going on for several decades but random structures of most scaffold proves to be a significant limitation. To mimic *in-vivo* environment scaffolds need to be highly oriented and Bio-MEMS can plays a vital role in solving this problem. Recent advances in micro/nanofabrication techniques have strongly impacted the tissue engineering field by precisely controlling the dimension, shape and structural uniformity of polymeric scaffolds. Surface microfabrication technology creates microenvironment by varying factors like surface topography, wettability, surface charge, surface proteins [33, 34]. Many efforts have been made to develop in-vitro cell culture systems, closely related to real cell microenvironment for cell behavioral study but more research is required to investigate the factor regulating cell behavior.

The same process of microfabrication used in silicon microelectronic chip manufacturing is used for fabrication of BioMEMS devices. These processes will be reviewed in the following sections.

2.1 Principle of Micro fabrication

2.1.1 Photolithography

Photolithography or optical lithography is the process of transferring geometric shapes on a mask to the surface of a silicon wafer. It uses light to transfer a geometric pattern from a photo mask to a light-sensitive chemical photo resist. The steps involved in the photolithographic process are wafer cleaning; photoresist application; soft baking; mask alignment; exposure and development; and hard-baking. Figure 2.1 explains photolithography process. This figure shows both types of photoresists, termed positive and negative.

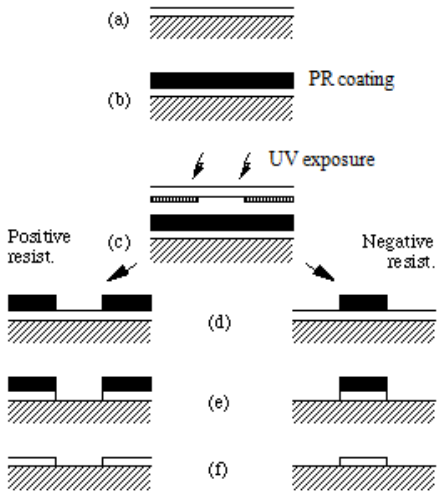


Figure 2.1 Lithography process by using both positive and negative PR with same mask.[35]

Ultraviolet light weakens the positive resist, so that when the image is developed the exposed resist is washed away - transferring a positive image of the mask to the resist layer. The opposite occurs with negative resist. The ultraviolet light strengthens negative resist polymer, so that when developed, unexposed resist is washed away - and a negative image of the mask is transferred to the resist [35].

2.1.2 Plasma etching

Plasma etching is a very useful tool to achieve high aspect ratio structure on silicon wafer. It involves a high-speed stream of glow discharge of an appropriate gas mixture being shot (in pulses) at a sample. The plasma source can be either charged (ions) or neutral (atoms and radicals). Ions are accelerated towards the target sample, and the etching reaction is enhanced in the direction of travel of the ion. Unlike anisotropic wet etching, plasma etching is not limited by the crystal planes in the silicon. The ability to microfabricate deep trenches in silicon substrates while maintaining high selectivity to masking material, good profile control and low nonuniformity across a wafer has made deep-reactive ion- etching (DRIE) one of the most important tools in the microengineering industry. As stated by Ayon et.al**.,** these DRIE machines can easily achieve etching rates in excess of 3 μm min^{-1}, selectivities to photomasking materials around 70:1 and non-uniformities across the wafer of 5% or less [37]. Most of these high density plasma tools are based on the Bosch process, also known as as pulsed or time-multiplexed etching. In the time-multiplexing scheme, the etching and passivating

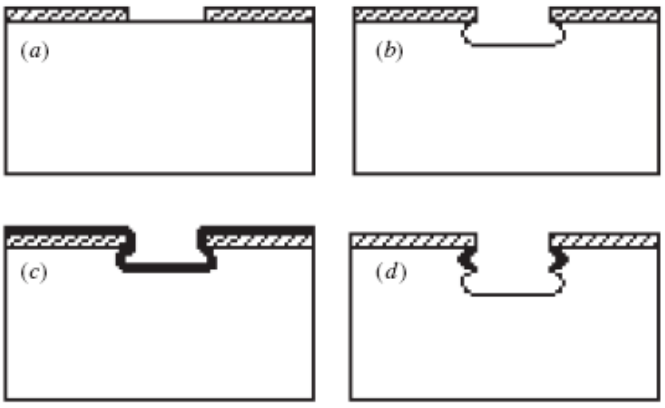


Figure 2.2 Diagrammatic sketch showing sequential steps during TMDE [37].

gases used are flowed independently one at a time and the machine alternates between an etching cycle and a passivating cycle unlike standard approach, in which all gas species are flowed at the same time. During the etch step a shallow trench is formed in the silicon substrate,

with an isotropic profile characteristic of fluorine-rich glow discharges. During the passivation cycle, a protective fluorocarbon film is deposited on all surfaces [37, 38]. Figure-2.2 explains the sequential of events that occur during time-multiplexed deep etching (TMDE).In (*a*) the masking material has been patterned. During the etch step in (*b*), a shallow, isotropic trench is formed. In the subsequent passivation step (*c*), a protective fluorocarbon film is deposited everywhere. During the next etch step (*d*), the fluorocarbon film is removed from all horizontal surfaces by directional ion bombardment, and another shallow trench is formed.

2.1.3 Soft lithography

Soft lithography represents an alternative set of techniques for fabricating micro- and nanostructures. In soft lithography, an elastomeric stamp with patterned relief structures on its surface is used to generate patterns, with feature sizes ranging from 30 nm to 100 μm. In the

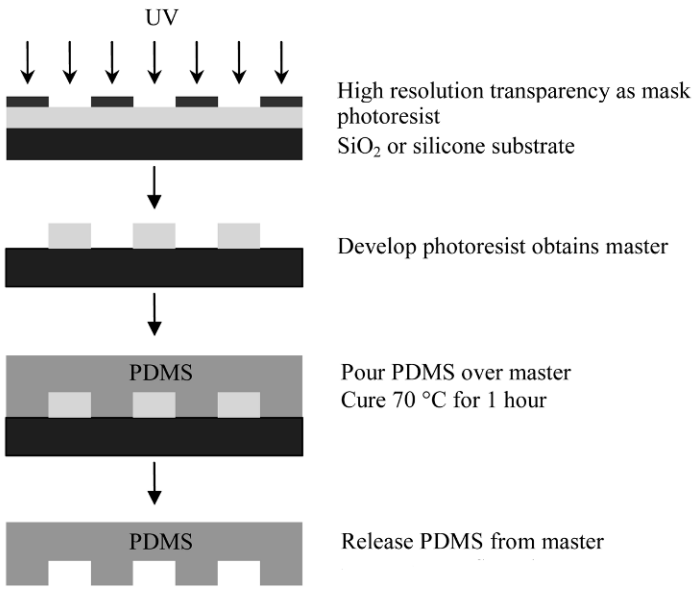


Figure 2.3 illustrated the process utilizes a silicone elastomer such as PDMS cast against a positive relief template to form high aspect ratio features. [39]

past few years this technique has offered a rapid and low-cost route to the creation of micro sized features on planar substrates [39].

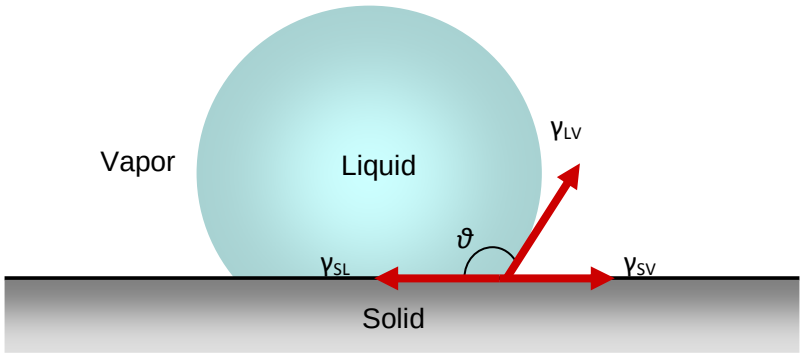
Out of different technologies used in soft lithography like Micro Contact Printing (μCP), replica molding (REM), microtransfer molding (μTM), micromolding in capillaries (MIMIC) and solvent-assisted micromolding (SAMIM), replica molding (REM) was used for our research purposes. REM is an efficient method for the duplication of the information (i.e. shape, morphology, and structure) present in the surface of a mold. Here we used both elastomeric and a rigid mold for pattern transfer. For high aspect ratio structures elastomeric PDMS mold were used because the elasticity and low surface energy of the mold allows easy peel of cured polymer [34].

2.2 Characterization of Microstructures

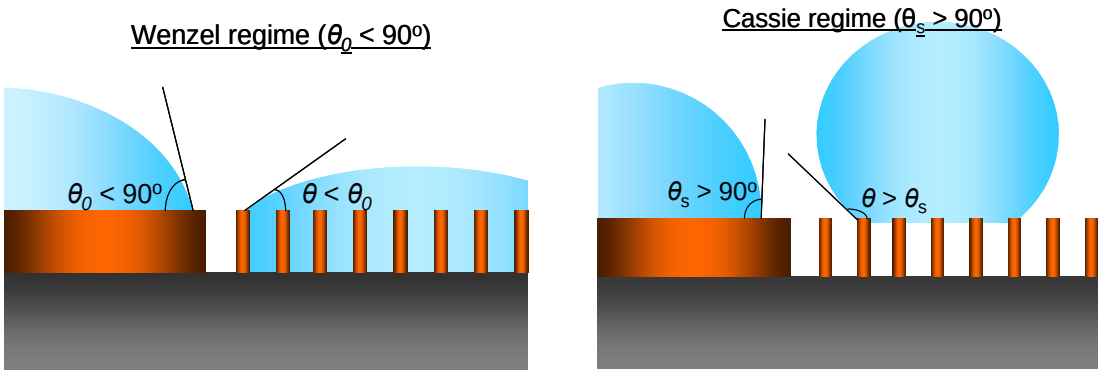
2.2.1 Contact angle measurement

The contact angle is a measure of wetting characteristics of the surface. A surface with a contact angle less than 90^o is called wetting or hydrophilic, and a surface with a contact angle over 90^o is called non-wetting or hydrophobic.

The contact angle is specific for any given system and is determined by the interactions across the three interfaces. If the surface is ideal (i.e., smooth, planar, rigid and homogeneous) the equilibrium contact angle is equal to the Young's angle that can be determined from the force balance between the interfacial tensions at the three-phase contact line formed by solid, liquid and vapor (Figure 2.4(a)) . However, non-ideal conditions of contact surface, affect the apparent contact angle therefore the Young's equation becomes invalid; an expanded approach is needed to describe contact angle of a droplet on rough surfaces that have composite interface. Cassie-Baxter and Wenzel are the two main models that attempt describe the wetting of textured surfaces. However, these equations only apply when the drop size is sufficiently large compared with the surface roughness scale [40].



(a)



(b) (c)

Figure 2.4 (a) Droplet on solid surface. (b) Wenzel regime. When liquid wets the solid surface, the area of the solid-liquid interface increases due to the surface roughness. Thus, the apparent contact angle becomes smaller than the intrinsic contact angle.(c) Cassie regime. When liquid does not wet the solid surface, liquid does not fill the structure and air pockets are formed beneath the liquid droplet. Thus, the area of solid-liquid interface decreases, and the apparent contact angle becomes greater than the intrinsic contact angle.

The contact angle θ_0 here is the intrinsic angle of a liquid on a flat homogeneous solid surface, while θ is the apparent angle on rough surface. γ_{lv}, γ_{sv}, and γ_{sl} are surface tension at liquid/vapor, solid/vapor and liquid/solid interfaces, respectively. Contact angles are measured by fitting a mathematical expression to the shape of the drop and then calculating the slope of the tangent to the drop at the liquid-solid-vapor (LSV) interface line.

2.2.2 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) is used for characterization of micro or nano range structures. The surface roughness is easy to estimate using SEM. The SEM is an instrument that produces a largely magnified image by using electrons instead of light to form an image because the SEM uses electromagnets rather than lenses, there is much more control in the degree of magnification.

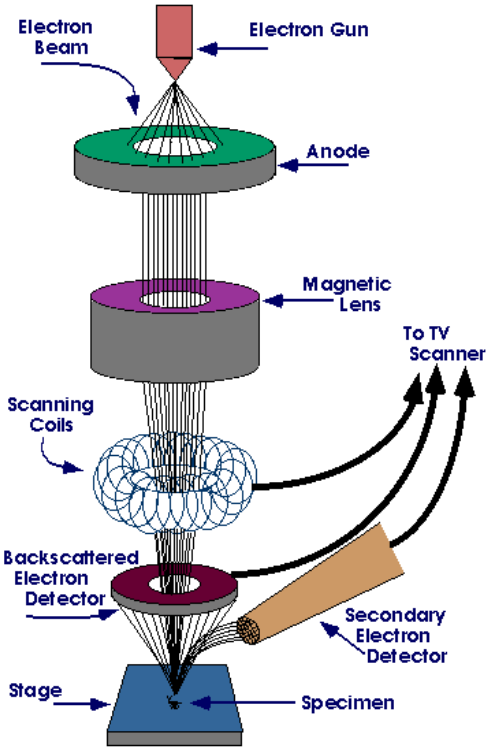


Figure 2.5 illustrate the concept of working mechanism of SEM [36].

A beam of electrons is produced at the top of the microscope by an electron gun. The electron beam follows a vertical path through the microscope, which is held within a vacuum. The beam travels through electromagnetic fields and lenses, which focus the beam down toward the sample. Once the beam hits the sample, electrons and X-rays are ejected from the sample. Detectors collect these X-rays, backscattered electrons, and secondary electrons and convert them into a signal that is sent to a screen similar to a television screen.

CHAPTER 3

MATERIAL USED FOR EXPERIMENT

The tissue scaffold provides a matrix for animal cells to grow in all three dimensions rather than spread out along a surface. The physical as well as chemical properties of the tissue scaffold have a tremendous impact on the growing cell morphology, spatial organization, proliferation, differentiation and functions. The need to therefore design and develop a single multifunctional material platform that can serve as a common controllable scaffold for the guided regeneration of different tissues is essential to regulate cell-cell and cell-matrix interactions

3.1 Scaffold properties for Implant

A number of materials or polymers design, have been experimentally studied. Ideally, a scaffold should have the following characteristics: (i) three dimensional and highly porous with an interconnected pore network for cell / tissue growth and transport of nutrients and metabolic waste; (ii) biodegradable or bioresorbable with a controllable (iii) suitable surface chemistry for cell attachment, proliferation, and differentiation; (iv) mechanical properties to match those of the tissues at the site of implantation; and (v) be easily processed to form a variety of shapes and sizes. In short, ideal scaffold material for tissue engineering application depends on the ability of the material to mimic the natural ECM and support cell growth [39, 41,42]. The material should have controllable degradation mechanism and high degree of immune tolerance *in vivo* [43]. For formation of small diameter blood vessel along with all characteristics for scaffold, mechanical strength plays an important role and it is hard to get mechanical strength conventionally used polyesters polymers like PGA, PLA, PCL and their copolymers being thermoplastic. Porous and fibrous scaffolds are inherently stiff and cannot be used as a scaffold where contractility is the basic requirement. Also, these scaffolds do not degrade completely [43,44] and offer poor cell alignment and morphology As mentioned above good scaffold

candidates for engineering SDBV should have good biocompatibility, which should be cell, tissue, and blood compatible. Ideal scaffolds should be non-thrombogenic and contribute for endothelium layer formation. The degradation rate of the scaffolds should approach the tissue growth or remodeling rate. Linear polyurethanes possess strong mechanical properties depending on the composition of their hard and soft segment. However, the degradability, biocompatibility, and susceptibility to permanent creep of polyurethanes still challenge their use for vascular regeneration.

For SDBV, a suitable biomaterial needs to perform mechanical graft functions such as support for the vascular cells, a temporary extra-cellular matrix. The desired biomaterial should perform analogous to a native blood vessel, having suitable strength, elasticity, and compliance [45].

3.2 Selection criteria for CUPE

To overcome aforesaid limitations of conventionally used polymers Dey *et al* developed Crosslinked urethane-doped polyester (CUPE). This polymer has combined properties of elasticity and biocompatibility and high mechanical strength because of presence of polyester and polyurethane groups. Increase elasticity coming from the increased crosslinking density of the polymer and this high ester bond crosslinking making the polymer biodegradable. Mechanical strength of CUPE improves because of doping of urethane compound which improves hydrogen bonding and provide a mechanically strong polymer. Its chemical and mechanical properties can be tailored depending on the application. Concentration of urethane bonds, polymerization condition and diol used for polymer are responsible factors for mechanical properties and degradation rate of CUPE. By changing these parameters the properties of CUPE can be changed. Material chemistry plays an important role in cell growth. Cell friendly material allows cell attachment and differentiation.

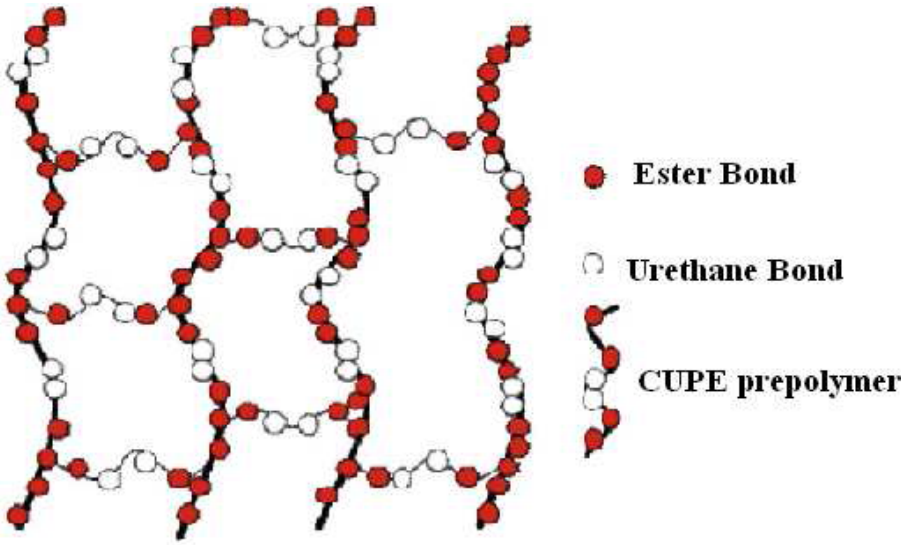


Figure 3.1 is diagrammatically explaining cross linked structure of CUPE network

CHAPTER 4

DESIGN AND EXPERIMENT SET UP

4.1 Mask Design

As mentioned above photolithography process transfers geometric pattern from a photo mask to a light-sensitive chemical photo resist. Therefore mask designing is the most basic and important step for microstructure fabrication. The design of masks for micromachining is usually straightforward. For our purposes, L-edit was used for mask designing. Once the mask design is complete, the files were converted in to CIF or GDS format as these formats are most commonly accepted by mask makers. The manufactured masks will be chrome on either low expansion glass, or quartz glass. Quartz glass mask was used as it transmits UV light better and is more stable.

4.2 Master mold preparation

For preparation of master silicon mold photolithography and DRIE was performed. Following steps were accomplished in fabrication of microstructures on the silicon wafer.

The process started with the cleaning of new wafer. The wafer was firstly cleaned with acetone and then by using Piranha clean (4 H_2SO_4: 1 H_2O_2) for 10minutes. After Piranha, wafer was rinsed with DI water and blow dried with nitrogen. After this dehydration step was performed. The wafer was baked at 195℃ using signatone hot plate for 10 minutes for dehydration. After that it was allowed to cool down for 1-2 minutes. Spinning was performed once dehydration of wafer was done. Firstly Wafer was spun coated with Hexamethyldisiloxane(HMDS) primer at 3000RPM/second for 30 sec with ramping at 500rpm/sec for 5 second and baked for 90 sec at 150℃ . After that photoresists SHIPLEY 1813 (S1813) was poured over the wafer surface with 95% of area was covered and spun coated at 4000 rpm/sec for 30 seconds at acceleration of 900rpm/s after a 5 second ramp at 500rpm/s at acceleration of 100rpm/second to get the 1.3

μm thickness. After this wafer was soft baked at 115℃ for 60 sec. Exposure was done using G Line OIA Aligner, wafer was exposed for 7.2 seconds to complete requirement of exposure dose of 140J/cm2. Post exposure bake was done on patterned wafer; wafer was hot baked at 110℃ for 60 seconds to harden the unexposed PR. After post exposure bake, wafer was put into developer MF 319 for 40-45 seconds with constant agitation every 10 sec to obtain transferred pattern by removing all exposed PR with UV light. For pattern transfer confirmation, wafer was visualized under the microscope available inside yellow room. In case of under development wafer was developed again for few seconds and confirm again, and in case of over development, all the first six steps till post exposure bake were repeated again to get the precise structure pattern. Once development is complete, wafer was hard bake to make the structures hard and stronger for deep reactive ion etching process. For DRIE, hard baked wafer was kept in DRIE machine for 5 minutes with etching rate of 2μm/min. For PR removal, hard baked PR was removed using PR stripper (BA355) at 60℃ for 5 minutes.

4.3 PDMS mold preparation

Using the Silicon microstructures obtained previously, polydimethylsiloxane (PDMS) microstructures were fabricated using a molding procedure. To fabricate the microstructure, the first step is to prepare PDMS mixture by mixing curing agent and PDMS monomer (SYLGARD® 184) in 1:10 ratio. In this process 4: 40 ml of curing agent and PDMS monomers were mixed respectively. This mixture was stirred constantly for 3-4 minutes to make sure that both the liquids had mixed properly. Then PDMS liquid was degassed for 15-20 minutes to remove all the bubbles from the mixture using a desiccator (NALGENE® labware).

Desiccator was connected to vacuum pump to create vacuum for PDMS mixture. In the mean while Sigma cote was spin coated over silicon microstructures that readily forms a covalent, microscopically thin film for easy peel off of PDMS from mold. Once all the bubble had been removed, this PDMS mixture was poured over the mold. This Silicon mold surface had the negative of the pattern that need to be transferred on to the PDMS device. The pattern on

PDMS device is created by pouring the liquid PDMS on mold and then heating in the oven (Quincy lab, Inc. ®) at 90° and 150° for 30 minutes and 10 minutes respectively. Once the PDMS get hardened, its surface now contains the desired pattern. The PDMS film was then peeled from the silicon substrate to obtain the reversed PDMS substrate.

4.4 CUPE scaffold preparation

Thin uniform polymer films were prepared by solvent casting of CUPE prepolymer on Silicon and PDMS molds. For that both the molds were cleaned with 70% ethanol to remove debris. To hold the desired volume of the polymer solution and to avoid the wasting of prepolymer solution, rectangular shaped containers were prepared using PDMS.

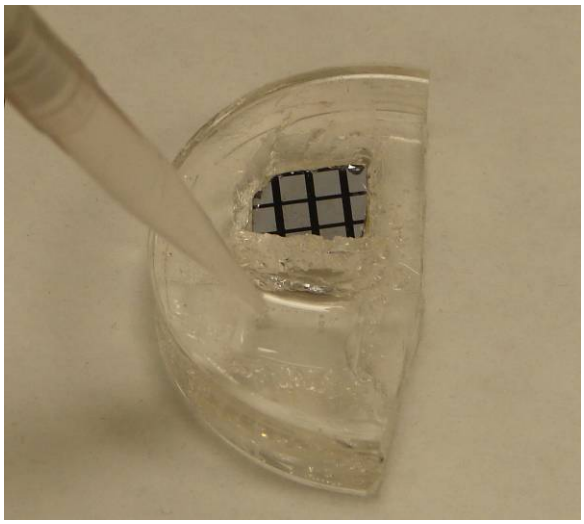


Figure 4.1 Image shows two chambers for CUPE casting, one with PDMS mold and other one with silicon microdome mold.

Figure 4.1 shows the CUPE holding containers, one with PDMS mold and anther one with silicon mold. PDMS containers were made because of their stability, multiple times usage, nonsticky nature for easy peeling of the polymer film, and most importantly to prevent any leakage or slippage of the polymer solution. Silicon and PDMS molds were glued to the container base and 3 ml of prepolymer solution was added into each PDMS container for 3 times The device were kept at room temperature to allow the solvents to evaporate from the

polymer solution. Once casting was complete, device was put into the oven at 80℃ for 48 hours. Once polymerization was done the complete device was dipped in ethanol: water solution for easy peeled off of polymer film from the molds. The CUPE films were peeled off with the help of forceps. These films were kept in freezer at -20°C for overnight and later vacuum dried to evaporate all water and ethanol from the films. The micro patterned structure transferred on the polymer film was confirmed firstly by observing the samples under microscope and later on with SEM.

4.5 Cell Culture Experiments

All cell culture experiments were performed were using fibroblast cells. Experiments were divided into two sections. Experiment A and Experiment B. Under “Experiment A” all kind of cell patterning and preferential cell growth experiments were conducted using micropillars and microdome structures, while in “Experiment B” alignment of cells were studied by using microchannels structures. Under “Experiment A” cell culture experiments were performed with both CUPE and PDMS Polymer and further subdivided in to four sections.

Experiment A(1)- Cell Culture on micropillars structures spin coated with thin layer of CUPE polymer.

Experiment A(2)- Cell culture on CUPE micropillars structures detached from silicon mold.

Experiment A(3)- Cell Culture on CUPE microdome structures.

Experiment A(4)- Cell culture on PDMS microdome structures.

Experiment B was conducted with CUPE polymer with microchannels structures only.

4.5.1 Cell culture on CUPE scaffold

NIH 3T3 Fibroblasts cells were seeded with cell density at a density of 7.2 10^4 cells/ml. Earlier cells were grown on the T 75 flask and were allowed to grow up to 80% confluency before trypsinization. Cells were passaged from the T 75 flask to CUPE samples in 96 well plate and put inside the CO2 incubator at 37°C and 5% CO_2. Dulbecco’s modified Eagle’s basal medium (DMEM) supplemented with 10% Fetal bovine serum (FBS) and 1% Penicillin- Streptomycin

was used for culturing. Cells were allowed to grow for three different time points 1, 3, and 5 days. After every time point samples were washed with PBS and fixed in 2.5% glutaraldehyde. For cell counting and visualization cells were stained with H&E.

CHAPTER 5

RESULT AND DISCUSSION

5.1 Structure Analysis

Various types of microstructures were fabricated on silicon and Su-8 mold using micromachining technology. The purpose of fabrication of all different kind of structures was to transfer the pattern over the biodegradable polymer. Transferring pattern using soft lithography techniques was a successful approach but to get the desired pattern and roughness, mold structures and the process was optimized by changing the parameters. Below tables will explain all kinds of molds fabricated at Nanofab, UTA.

Table 5.1 Table lists the Silicon mold, PDMS and CUPE microstructures

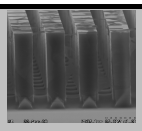
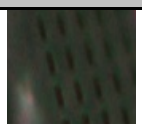
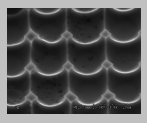
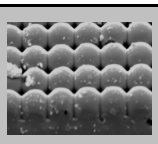
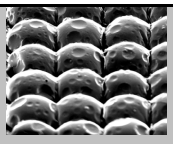
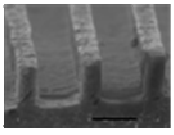
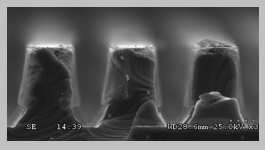
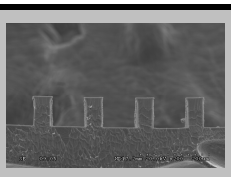
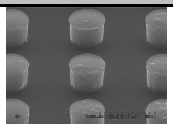
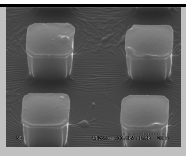
Silicon Mold	**PDMS Mold**	**PDMS Microstructure**	**CUPE microstructure**
	Detachment issues	---	---
	Not Required; CUPE tried molded from Si mold directly	Detachment issues	Detachment issues
	Not Required; CUPE molded from Si mold directly		
Silicon channels (20μm)	PDMS mold fabricated	---	

Table 5.1 and table 5.2 give the brief idea of unsuccessful and successful attempts to obtain structures from the Si and SU8 molds. It was much easier to obtain structures form SU8 mold as compare to silicon because of lower aspect ratio structures and less sticky nature of surface but for our research purposes, to pattern biodegradable polymer either SI mold or PDMS mold was required as direct casing of polymer on SU8 was advisable. Therefore SU8 mold was used for limited purposes.

Table 5.2 lists the SU8 mold, PDMS mold, PDMS microstructure and CUPE microstructures

Su-8 mold	**PDMS mold**	**PDMS Microstructures**	**CUPE Microstructures**
SU-8 channels(80μm)		---	
SU-8 micro circles	---		---
SU-8 micro squares	---		---

5.1.1 Molds and Pattern transfer

Using micromachining Silicon micropillars were successfully fabricated and structures were analyzed using SEM microscopy as shown in figure 5.1. Very high Aspect ratio structures were obtained with specifications of Pillar height: 38μm, pillar width: 7μm and space between pillars: 6μm. By using the same process but photomask with opposite polarity, microholes were created inside silicon wafer. Both patterns gave difficulty in detaching the polymers from the mold. Possible reason could be high aspect ratio and increased stress concentration at edge.

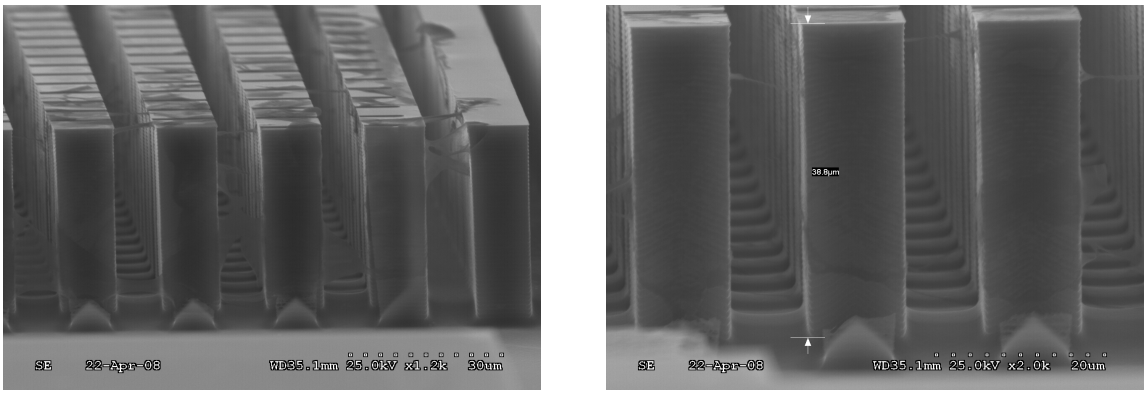


Figure 5.1 SEM images showing the micropillars structures fabricated over the silicon wafer.

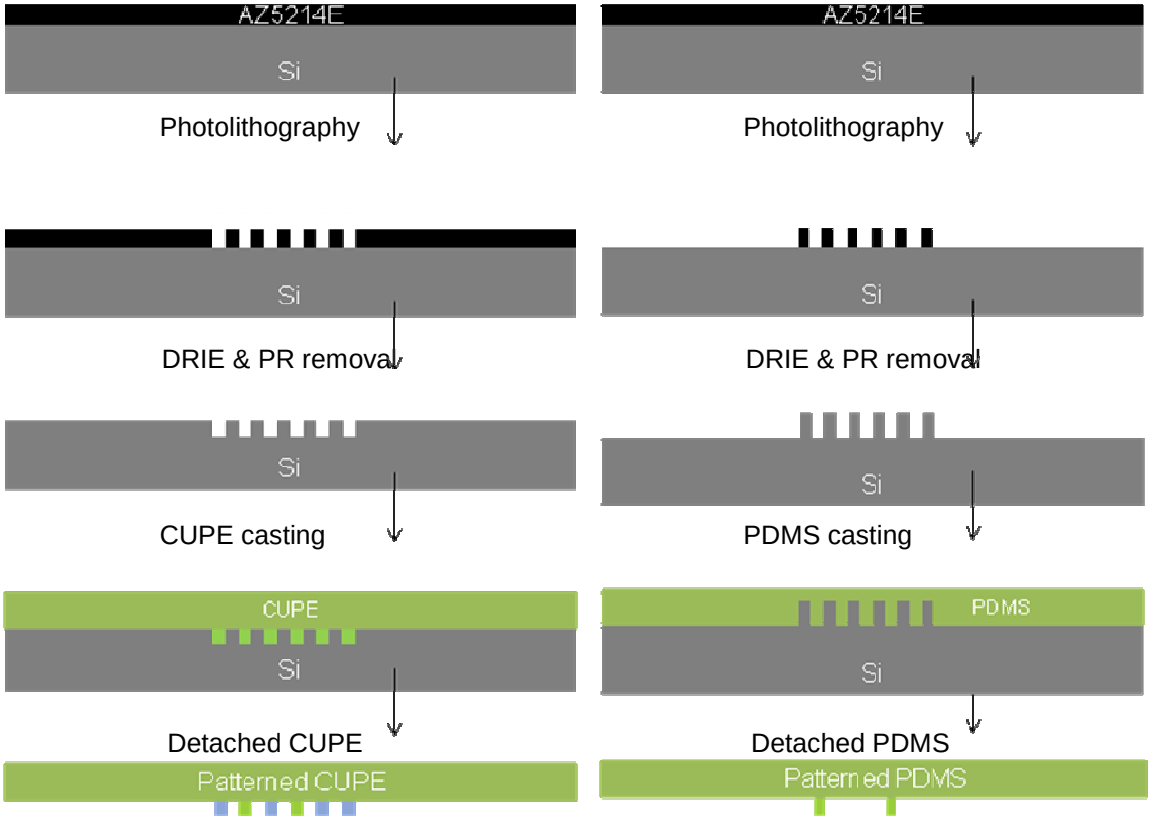


Figure 5.2 Sketches illustrate the difficulty in patterning the polymers while demolding it.

Microtransfer molding processes of CUPE and PDMS microstructures are illustrated in figure 5.2.Circled region shows the damages area on CUPE and PDMS.

As mentioned above demolding of CUPE as well as PDMS was big issues because of sharpen edged pillar structure and their aspect ratio. Although it very was difficult to detach CUPE polymer from the Silicon mold, but we were able to get small section of micropillar patterned CUPE polymer from the silicon microholes mold.

That micropillar patterned CUPE polymer was used to study fibroblast cell response over the patterned polymer, which will be discussed later in this section. Major challenge was to come up with a new design, where cell behaves similar to micropillars structures but make polymer peel off process much easier. For that purpose we came up with microdome structures. Dome structure were much easier to peel off because factors such as sharp angles and high aspect ratio present in previous molds, which leads to increases stress concentration and results in polymer tearing were absent. In addition to structure design change SIGMACOTE, a chemical was also used for easy detachment of polymer.

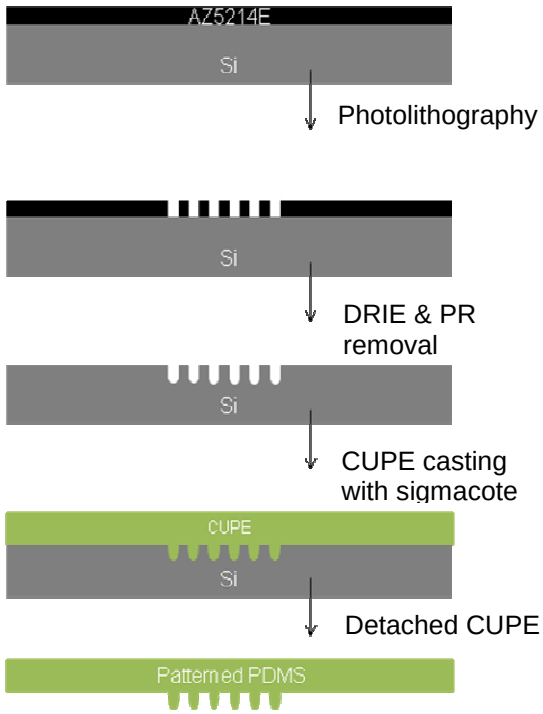


Figure 5.3 Image shows the fabrication of microdome structures and pattern transfer over the CUPE polymer.

Sigmacote is a special silicone solution in heptane that readily forms a covalent, thin film over mold, and allows easy detachment of CUPE. Figure 5.3 shows diagrammatic view of the process for microdome fabrication on silicon wafer and successful transfer of the pattern to CUPE polymer.

Below Figure shows the SEM images of successful microdome mold pattern over silicon wafer

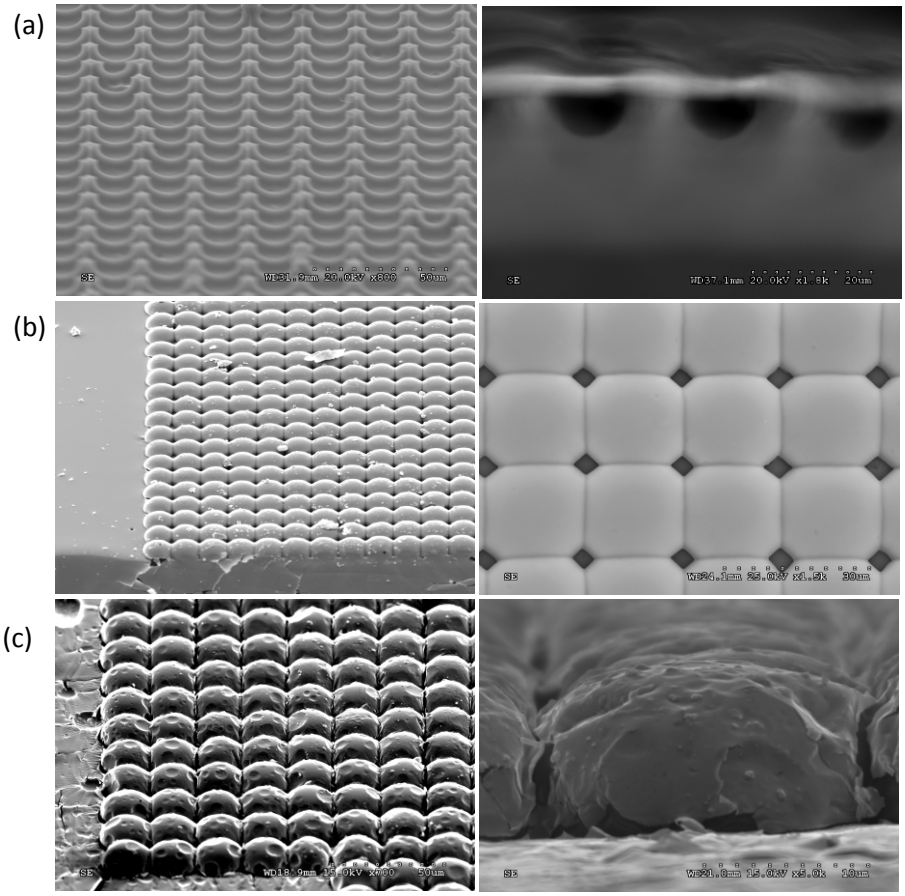


Figure 5.4 Image shows the dome shape structure mold for (a)Silicon, (b)PDMS microdome structures and (c)CUPE microdome structures.

and pattern transfer PDMS and CUPE polymer. SEM images of microdomes show the uniform pattern transfer over the PDMS and CUPE polymer. Silicon mold depth is 10 μm and diameter is 15μm and the corresponding PDMS and CUPE dome diameter is15μm and height is 10μm.

Micropillars and Microdomes structures were used for preferential cell growth over smooth surface than patterned surface. Detailed Cell culture results on these structures will be discussed later in this section. Microchannels structures were fabricated to align the cells unidirectional to fulfill the objective to this project. For easy peel of CUPE patterned polymer PDMS mold was used as intermediate mold.

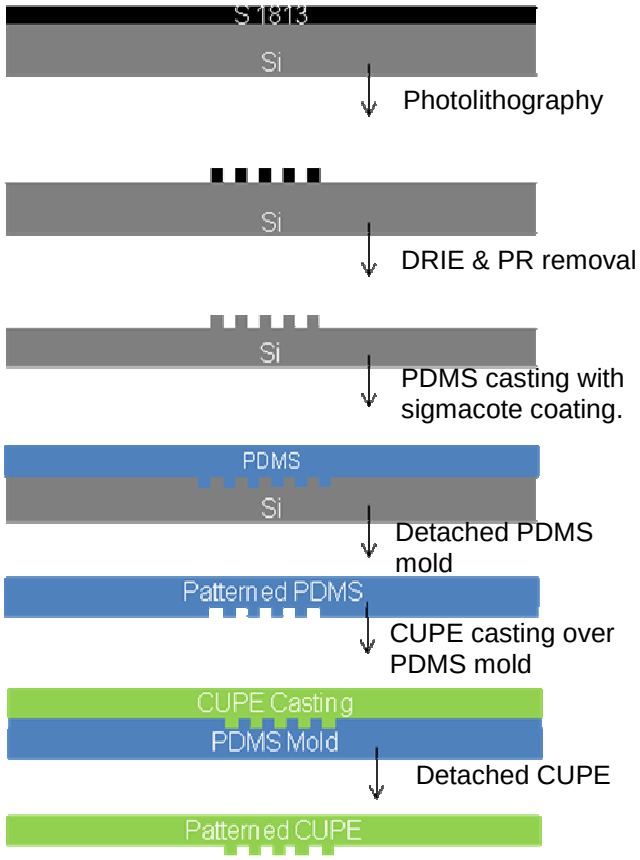


Figure 5.5 This Image illustrates the process of transferring pattern from PDMS mold to the CUPE polymer.

Figure 5.5 shows the process of patterning channel on CUPE. SU8 and silicon were used as molds for preparation of PDMS molds. SU8 mold was used to fabricate 80-90μm deep channel structures, while silicon mold was used for 20μm deep channel.

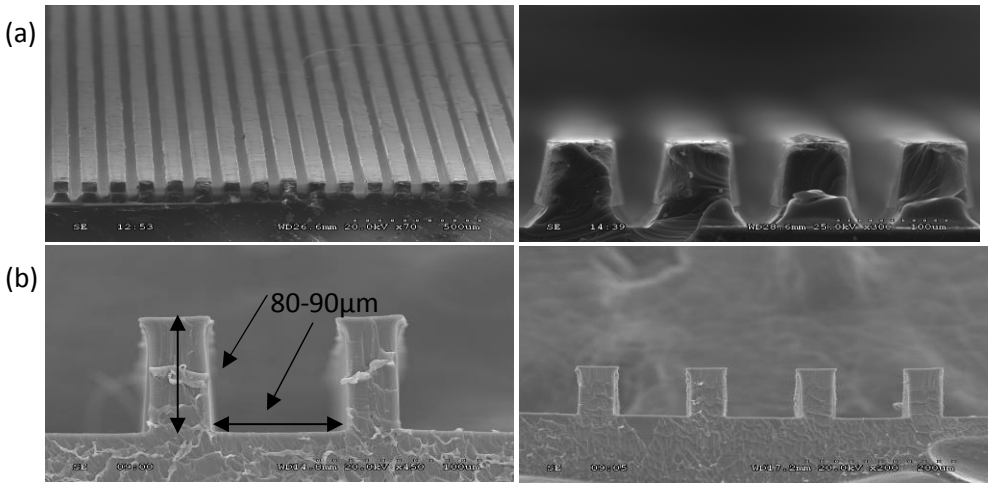


Figure 5.6. This Image illustrates (a) PDMS channels mold and (b) CUPE channel micro structures with very high aspect ratio.

Figure 5.6 shows the channel pattern transfer over the PDMS mold and then to CUPE polymer. But because of very deep structures and huge air gap between channels, cells were not able to settling down in channels therefore another set of channels were fabricated on silicon mold with less deep structures. Figure 5.7 shows the final CUPE patterned polymer from the PDMS mold obtained from the Silicon 20µm deep channel structures.

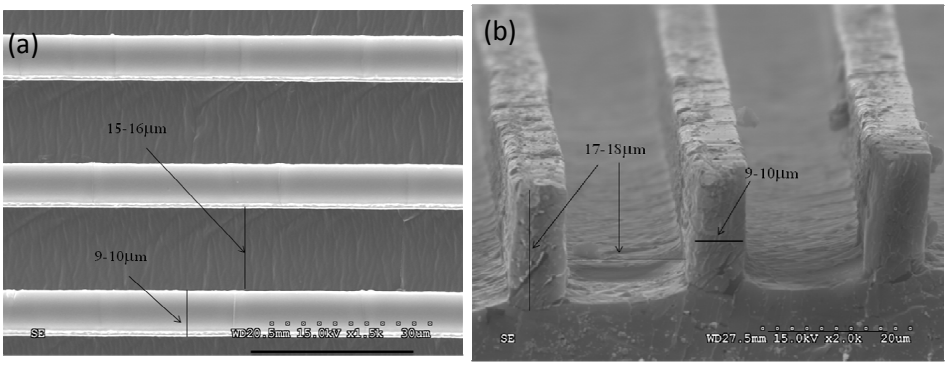


Figure 5.7 Top view (a) and side view (b) of CUPE micro channels structure.

Culturing cells over the above channel structure gave expected results with almost 80% of the cells were found aligned along the channel. Cell culturing results will be discussed in detail in next section.

5.2 Cell Culture results

All cell culture experiments were performed were using fibroblast cells. Experiments were divided into two sections. Under “Experiment A” all kind of cell patterning and preferential cell growth experiments were conducted using micropillars and microdome structures, while in “Experiment B” alignment of cells were studied by using microchannels structures.

5.2.1 Experiment A

Figure 5.8 shows images for first set of experiment A(1) were CUPE was spin coated over the silicon microstructures. This experiment was done for proof of concept and the data showed cells were differentiating between the surfaces. The sample has both smooth area and micropillars-structured rough area. A thin layer of CUPE was spin coated over the sample.

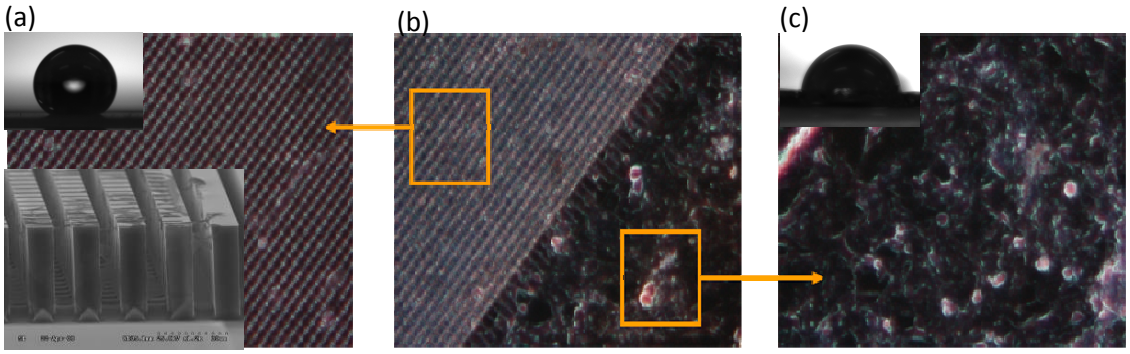


Figure 5.8 Interface (b) between the patterned and smooth surface. (a) & (c) are the insight of cell culture area. Smooth surface(c) is completely populated with cells on the other hand rough surface (a) have very few number of cells. There is clearly a huge contact angle difference between image (a) & (c).

Water contact angles on different surfaces on the sample were measured. As shown in Figure 5.8 water contact angle on microstructured polymer surface is much greater (superhydrophobic) than that on the smooth polymer surface. The sample was subjected to 3T3 Mouse Fibroblasts. Cell culture process with cell density of $1x10^6$ cells/mL for 3 days. These results showed cell tends to stay away from superhydrophobic surfaces and preferably grows on the smooth surface. Experiment A(2) was to fabricate microstructured scaffold, micropillars patterns were transferred to biodegradable polymer by the method of microtransfer molding (Figure 5.9).

Silicon mold and microstructured biodegradable polymer with transferred patterns are shown in Figure 5.9(a) and contact angle measurements are shown in Figure 5.9(b) and (c). The same cell culture process has been followed on scaffolds sample with both microstructured and smooth area. Figure 5.9(b) shows no cells grown on microstructured CUPE while Figure 5.9(c) shows many cells are proliferated on smooth CUPE surface. Note that two different surfaces

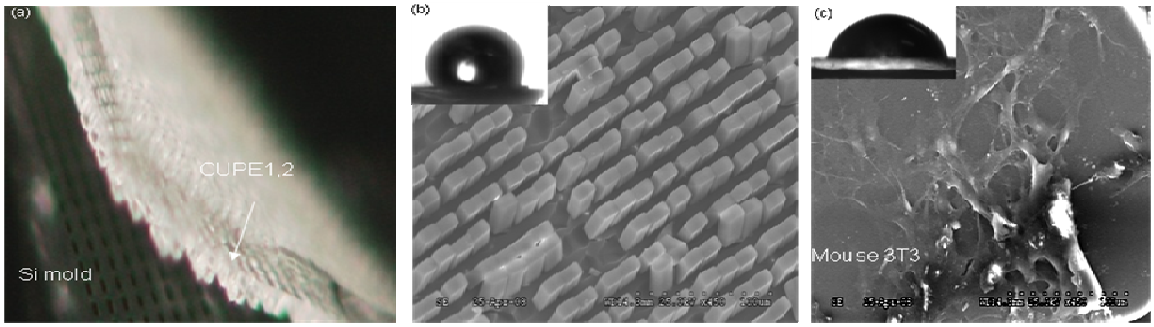


Figure 5.9 3D micrograph of microtransfer molding. CUPE1,2 microstructures are successfully transferred from Si mold. (b, c) SEM image of CUPE1,2 after 3 days cell culture.

are on the same sample chip, thus both surfaces were under the same cell culture process. Contact angle measurement was 105° and 81° respecti vely on patterned surface and smooth surface. Figure 5.9 (b) microstructured area with no cell grown, and (c) smooth flat area with proliferated 3T3 cells As mentioned earlier, this process of transferring pattern form Silicon mold to CUPE was hard, Therefore low aspect ratio structure needed to be fabricated, form which CUPE is easy to peel off but also provide same degree of hydrophobicity as the micropillar structures. Experiment A(3) was conducted with CUPE microdome shape structures. Microdome patterns were also transferred to biodegradable polymer by the method of microtransfer molding. Silicon mold and microstructured biodegradable polymer with transferred patterns are shown in Figure 5.4(a) and contact angle measurement on this pattern was found to be very similar (106° and 80°) to contact angle to pillar structure. Similar results were observed with microdome structures on the PDMS (Figure 5.11). This experiment A(4) was conducted to verify the cell behavior with dome structure and also to confirm the cell growth with PDMS as It was used for studying real time monitoring of cell culture behavior on the microfluidic chip.

Real time monitoring of culturing will be discussed on more detail in the next chapter.

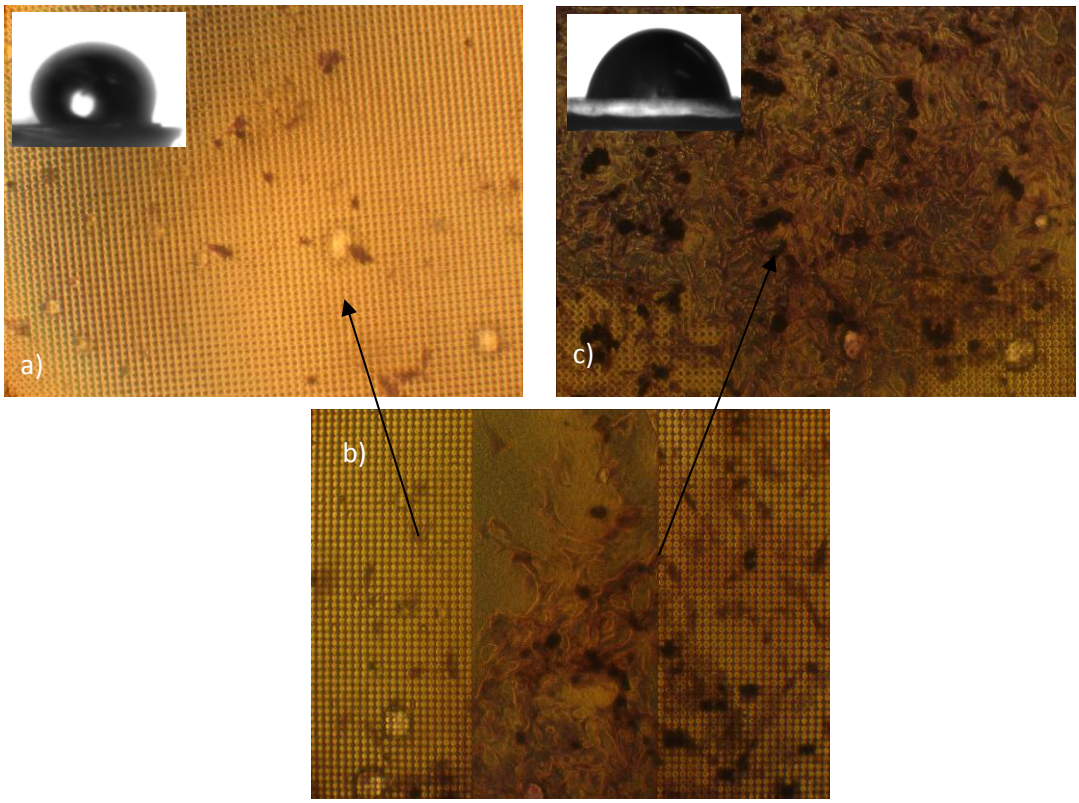


Figure 5.10 Cupe paterned with microdome structures. Image (b) is showing the interface between the patterned and smooth surface. (a) & (c) are the insight of cell area.

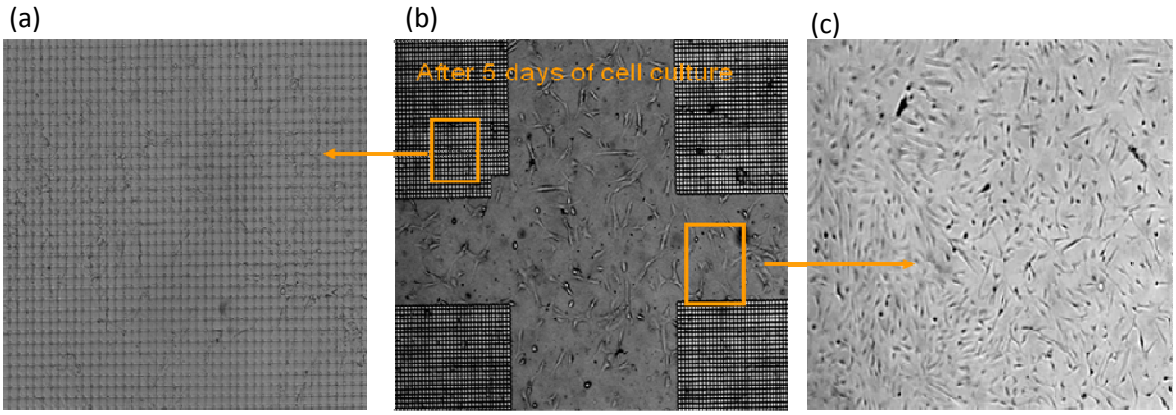


Figure 5.11 Five day Cell culture results with dome shape structures on PDMS.

All the above results conclude that cells are clearly differentiate between the patterned and smooth surfaces, Much higher density of cells was found on smooth surfaces as compare to patterned surfaces.

5.2.2 Experiment B

Experiment B was performed with channel structure pattern for the guided cell growth.

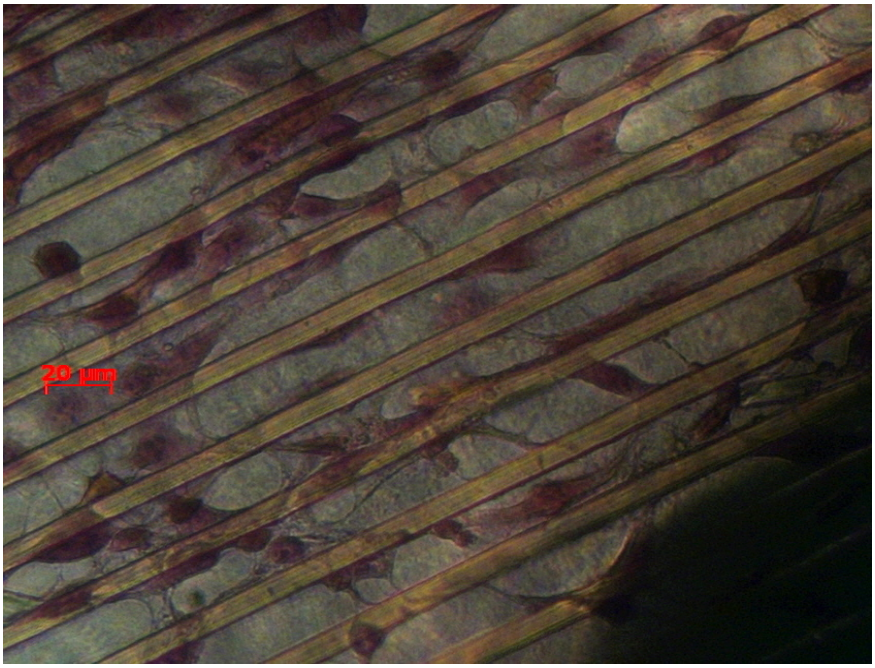
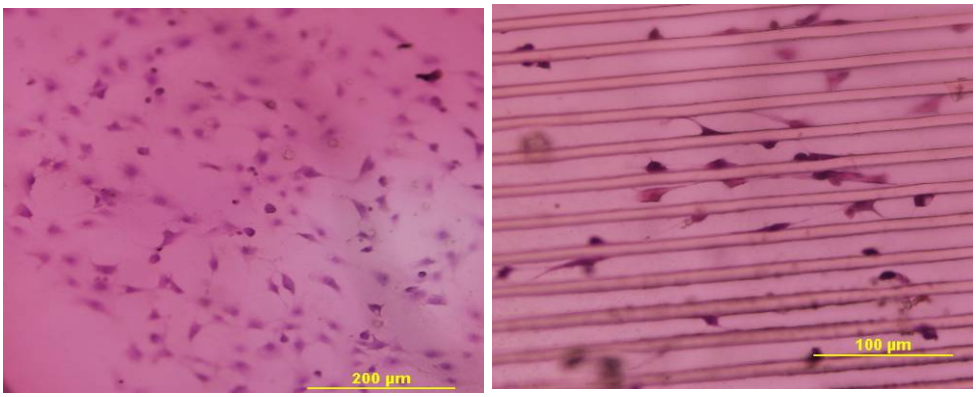
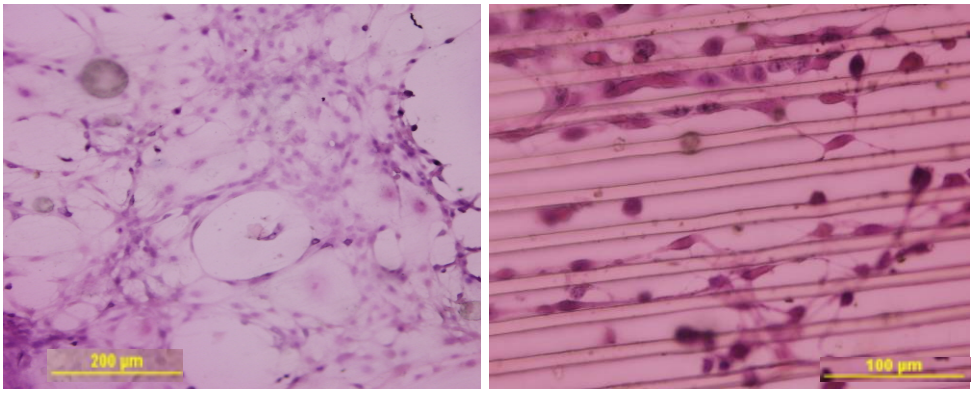


Figure 5.12 shows the clear alignment of cells along the channel structure on day 3.

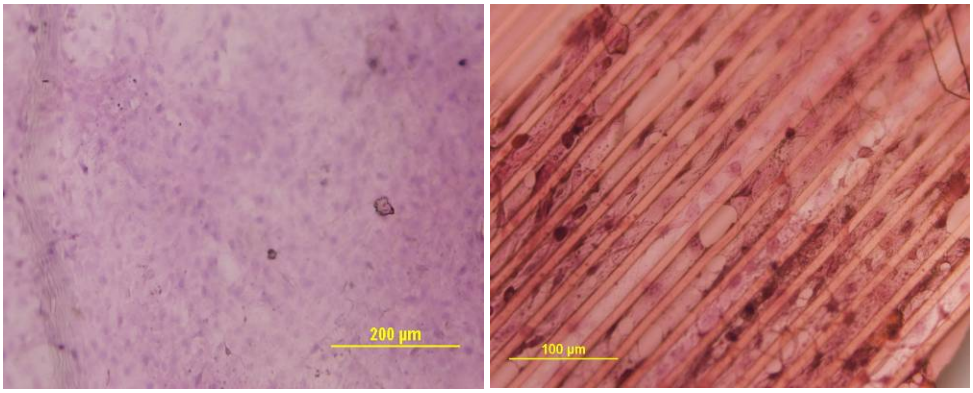
H & E staining of the samples illustrate difference in morphology of the cells between control samples and treatment samples. Figure 5.12 clearly shows cells are growing along the wall of channel. Study was performed for five days, H & E stained images illustrate obvious difference in alignment of the cells between control samples and treatment samples. H & E staining of the samples reveals alignment in the samples. In day 1, 3, 5 all samples as shown in figure 5.13 cells are arranged parallel to the grooves and most of them are confined in the grooves. While the sample without grooves (control) shows random arrangement of the cells.



Day 1



Day 3

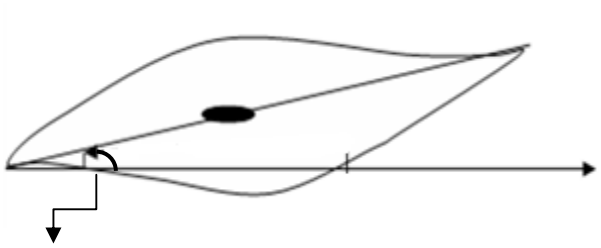


Day 5

Figure 5.13 shows the 5 days study of fibroblast cells on control and microchannel pattern.

Quantification

Using Image J orientation of the cells was quantified. For this quantification also 250 cells were selected for day 3 and day 5. The angle of the major axis of the cells with respect to a reference line was measured.



Angle made by major axix with reference line.

Figure 5.14 Cell alignment angle with reference to the channel axis.

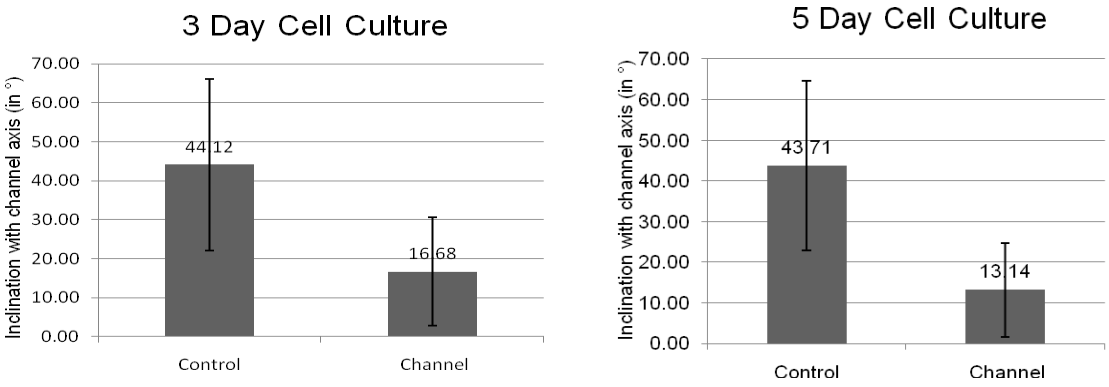


Figure 5.15 Graphs show the average alignment of cells on control and channel samples for 3 day and 5 day study.

Data shows that on day 3 on average cells are aligned with channels at 16.68°, and on day 5 with an angle of 13.43°. While the control for both the days are randomly aligned between 43°-46° with the same axes of channel. Distribution of cell population data shows that almost 80 % of cells are aligned with in 0-20°range. The cells lying in this range were defined as aligned, while the cells lying in the range greater than 20° are defined as randomly oriented. The graph of angle vs. % of cells for 3 day and 5 day was plotted as shown in the next page.

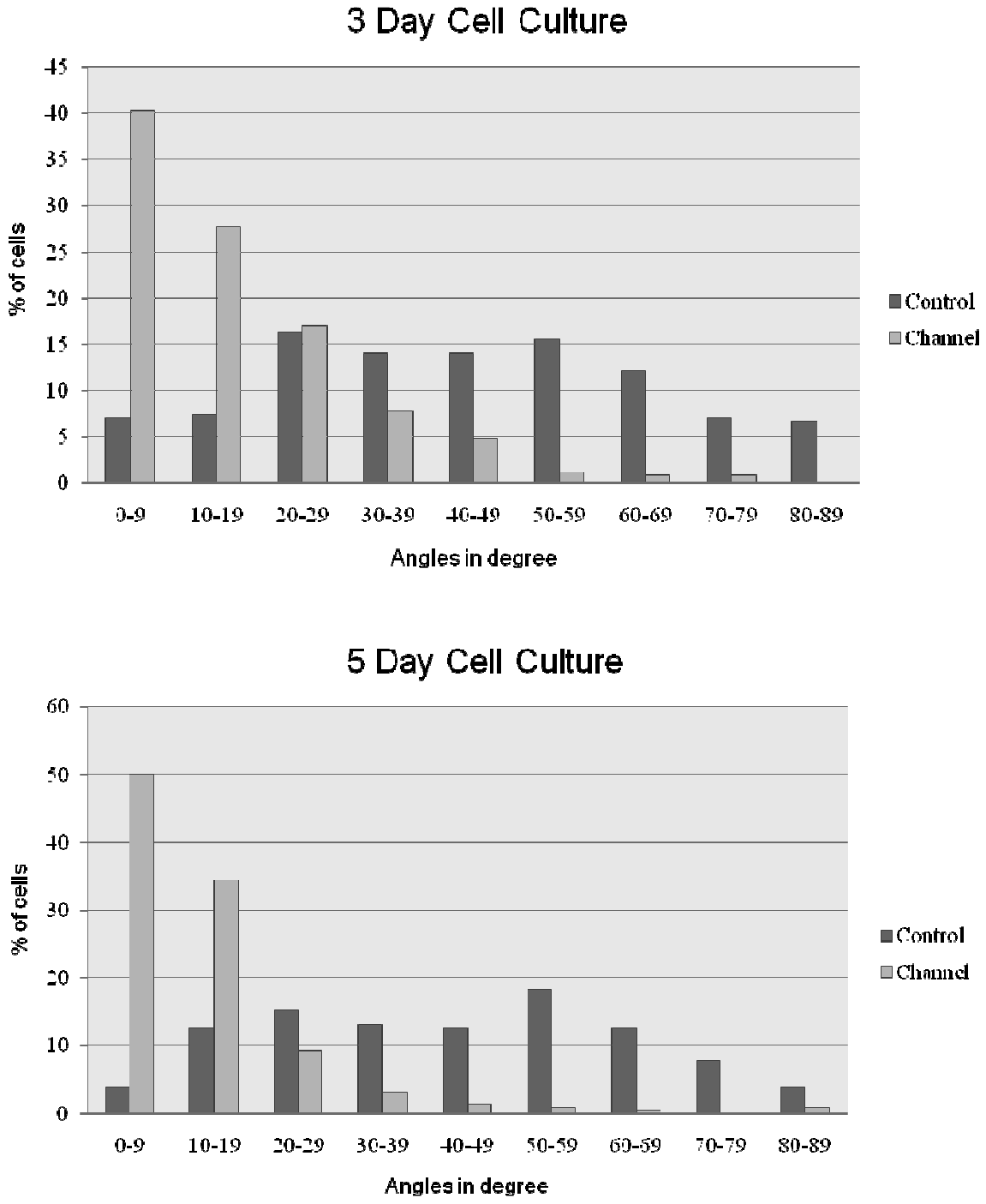


Figure 5.16 Distribution of alignment in cells on CUPE film with channels and control for 3 day and 5 day cell culture.

Figure 5.16 clearly demonstrate almost all cells in channel structures were skewed to the left side of graph, while in control samples cell population is uniformly distributed. These graph shows in control cells are randomly oriented without any preferences.

CHAPTER 6

MICROFLUIDIC DEVICE FOR REAL TIME MONITORING OF CELL CULTURE

6.1 Introduction

Various studies have explored the use of microfluidic devices to culture cells. Such studies show that microfluidic systems have high cell culture efficiency, high reproducibility, and are better at mimicking the in-vivo environment which cells are exposed to. All of these factors are highly desirable for the purpose of studying the effect of microstructures on cell culture behavior. While studying cells response to microstructures, it is important that an environment similar to cells in-vivo environment be maintained, so that any discernible behavior of cells can be attributed to the effect structures, rather than other unregulated factors or mismatch in environment. Using microfluidic cell culture systems allows precise control over the environment of the cells, Cells can be subjected to compression, shear stresses and even pulstative flow, all of which is required to match an in-vivo environment and very difficult to create in a conventional cell incubator. This makes the use of microfluidic devices highly desirable in studies of structures behavior on cells, especially in the larger context of creating complex tissue scaffolds where such tissues will be exposed to various stresses and flows that exist in their natural environment in the body. The microstructures that are used are easily integrated into the cell culture chambers during the fabrication of microfluidic cell culture systems. The high reproducibility of these devices also ensures that such devices are identical to each other, when multiple devices are used for various experiments. This ensures that the data obtained is reproducible and the results obtained are consistent [46, 47].

As stated in previous chapter, cells react to structures in different ways. The best way to study the cell response to structures is by means of a real time experiment wherein the cells can be

viewed as they are seeded onto surfaces and aligning or migration of cells over time can be seen and correlated with the kinds of surfaces used.

The aforementioned novel technology can be applied to many applications in the field of biomedical engineering, such as guided blood capillary formation and avoiding restenosis, inflammation, thrombosis, and other serious complications associated with cardiovascular interventions. However, important questions still remain unaddressed: how cells can distinguish rough surfaces from smooth surfaces; how cells behave and respond to the surface structure; and what type of structures prove most effective in orienting and aligning cells, maximizing cell densities, maintaining cell differentiation, and improving cell function. Therefore, here we developed a microfluidic platform for cell culturing to address these unanswered questions.

6.2 Objective of research project

The purpose of this study, to design a real time device that will allow visualization of cells over initial cell attachment phase, and for longer periods of time thereafter to observe cell alignment and migration. Conventional real time cell monitoring systems utilize specialized expensive transparent incubators attached to microscopes that are capable of imaging cells inside the incubator. By using microfluidic cell culture systems as mentioned above, a real time study can be conducted by visualizing the microfluidic cell culture device under any ordinary inverted microscope. This allows multiple experiments to be run simultaneously without the use of costly specialized equipment, and also, can yield more accurate results since the advantages of microfluidic cell culture devices over incubators have already been discussed. Thus, the objective of this study is to design and fabricate a microfluidic cell culture device with integrated structures that can be utilized for real time cell culture studies.

6.3 Design and Experiment

The real time microfluidic device consists of 3 PDMS layers bonded together. The bottom most layer was pattern with microstructures. As mentioned in previous chapters microstructures were created by casting PDMS over Silicon micro-patterns. Once cured and removed from the mold,

the outer 4mm of the 8mm circular cell culture chamber area was spun coated with 1% Teflon at 1500 rpm for 30 seconds. After spin coating, Teflon was cured in the oven at 150° C for 3 hours. This bottom layer of Teflon coating creates an outer hydrophobic layer which discourages bubbles from intruding into the inner 4mm of the cell culture chamber which was the area under observation.

The middle layer of the device was obtained by casting a 3.6mm sheet of PDMS. The cell culture chamber in the middle layer was made by using an 8mm biopsy punch to cut a through hole in the middle layer. The inlet and outlet holes were made as well using a 3mm biopsy punch. The inlet and outlet holes are connected to the cell culture chamber by channels that are cut into the PDMS layer as seen in Figure 6.1

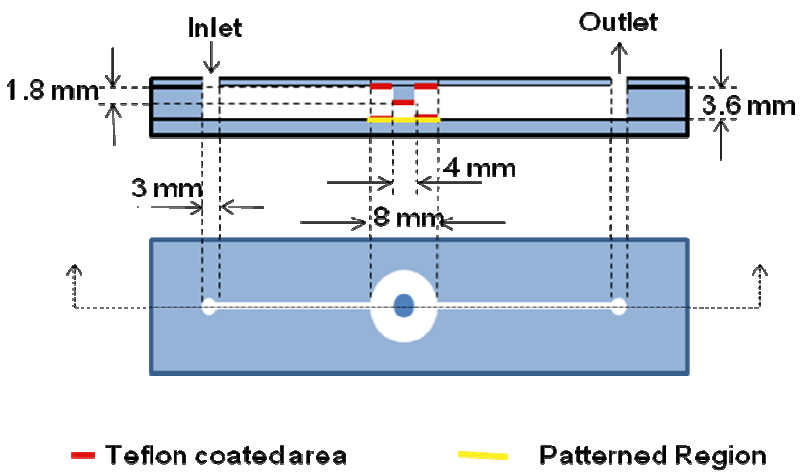


Figure 6.1 Microfluidic device sketch for bubble trap design with parameters.

The top layer was another PDMS sheet which had a 1.6mm thick extended step height with 4mm diameter, which fits over the centre of the cell culture chamber area when assembled. Once cured, inlet and outlet holes were punched to correspond with the inlet and outlet of the middle layer of the device. Teflon was spin coated over the 8mm portion of the top device which fits over the cell culture chamber area. This top Teflon coating encourages bubbles to remain over the top of the device rather than attaching to the bottom. It also prevents bubbles from attaching to the surface so that during perfusion, bubbles get flushed out of the system.

The three layers were bonded together by treating them with Corona treatment for 15 seconds and then they were pressed together and placed in an oven at 60° C for 1 hour. This yielded a strong bond between all the layers, the integrated device was obtained.

6.3.1 Real Time Cell Culture Experiment Setup

To maintain cells physiological condition constant temperature and pH control is required. For this purpose, a heater was constructed using ITO glass. ITO glass was choose as the heat source generator because of its optically transparent property, which will allow the real time observation of cell culture on chip. ITO glass was bonded to copper tape by means of silver epoxy and the copper tape was attached to the bottom of a Petri plate. Sourcemeter was used for constant current source to maintain 37.4° C ins ide Petri plate.

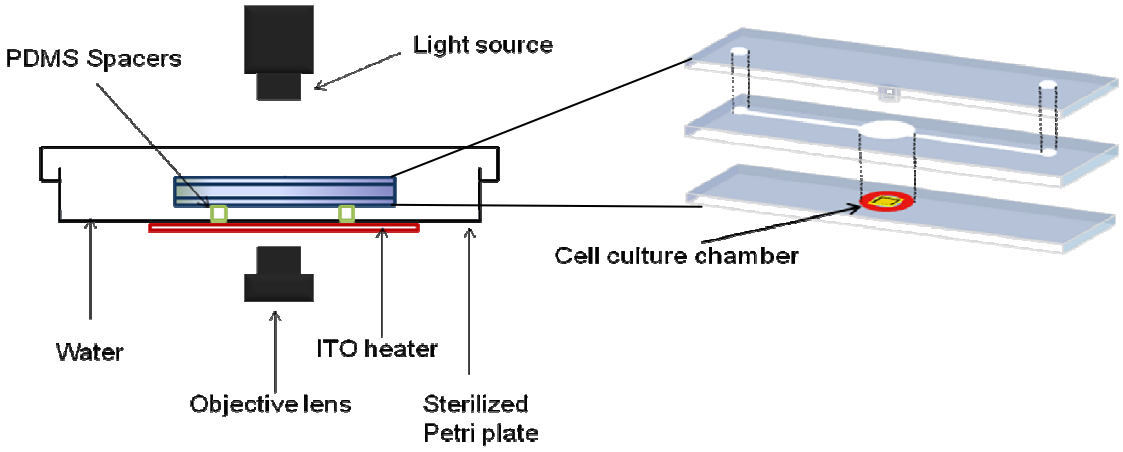


Figure 6.2 Diagrammatic view of real time device setup and Insight of 3 layer microfluidic cell culture system

Figure 6.2 explains the setup of real time device. The Petri plate was filled with 12 ml of water which helped in maintaining the 95% humidity environment and also aided in uniform distribution heat across the Petri plate. Once the microfluidic device had been seeded with cells, it was placed in the Petri plate and kept above the water by means of glass spacers. The Petri plate is covered and placed under an inverted microscope for observation for real time cell culture study. All preparations of cell culture on the microfluidic chip were performed in a laminar hood to prevent contamination.

6.3.2 Cell culture on real time device

Human dermal fibroblasts (BR5) cells were seeded with cell density of $1.0x10^5$ cell/ml. Advanced Dulbecco's modified Eagle's basal medium (DMEM/F12, Sigma, Missouri, USA) supplemented with 5% Fetal bovine serum (FBS), L-glutamine, Penicillin, and Streptomycin was prepared for culturing the cells.

6.4 Results and discussion

Fibroblast cells were cultured in microfluidic devices described above. The first 4 hours of cell culture was done as a real time study under an inverted microscope. Figure 6.2 Images were taken at 30 minute intervals and fibroblast attachment could be seen to occur within 180 minutes. After four hours, the microfluidic devices were placed in the incubator and a follow up incubator study was done for five days. Figure 6.3 Images were taken each day and the cell density was found to progressively increase. This clearly shows that the initial four hours of real time study was viable for cells and those cells were still alive and proliferating.

During the four hour real time study, attachment of fibroblasts could be seen but no clear migration could be observed from patterned area to smooth surface. However difference in the cell attachment was observed, with more cells being seen on the plain areas and less on the dome shaped structures that were used to create patterns. The cells that were seen on the dome shaped structures did not appear to elongate as is characteristic of fibroblasts when they start attaching to surfaces. This seems to suggest some preference of cells for plain areas, over dome shaped structures. This was further reinforced during the follow up incubator results with increased cell density on real time study.

During the real time study, it was seen that some cells retained their round shape even after four hours. The lack of change in morphology indicated likely cell death. Thus although cells did survive during the real time study, the overall viability of cells was closer to 60%. To make this real time device conditions exactly to incubator there exists further scope for improvement.

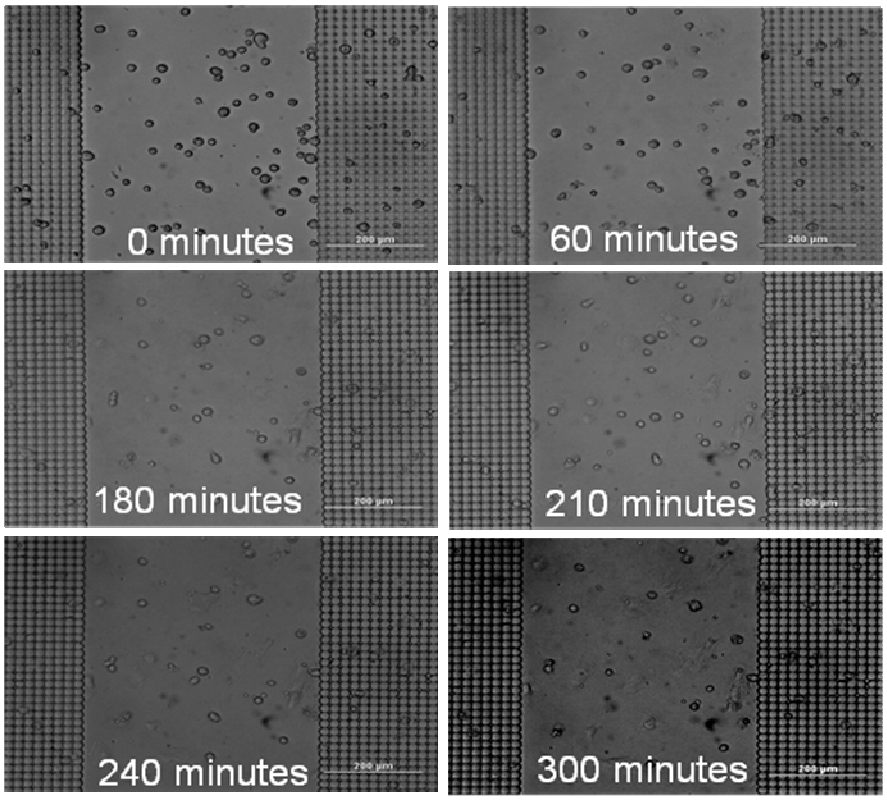


Figure 6.3 Cells were cultured without conventional incubation systems. This shows the real time attachment of fibroblast cells. Images were taken at 30 minute interval

Although temperature control and bubble problem have been fixed in the existing setup, there is no means for maintaining CO_2 and pH balance. Clear areas while structured areas seemed to be show very little cell extension.

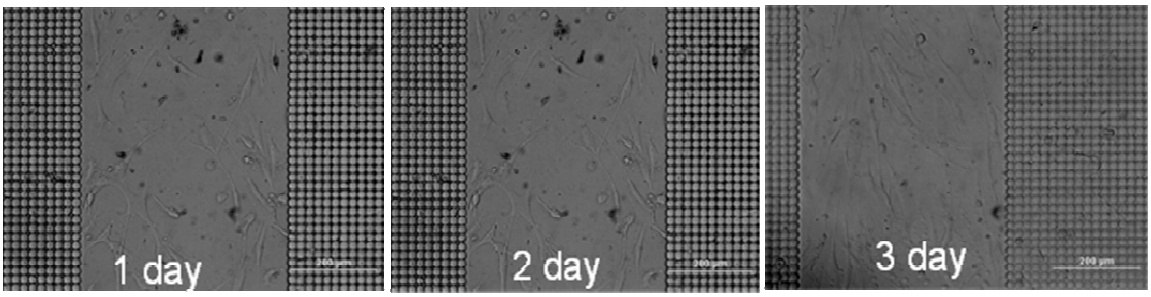


Figure 6.4 Microfluidic chips were put inside the incubator for 3 day study after completion of real time study.

The lack of CO2 and pH balance could explain the cell death that was observed during the 4 hours of real time study, and is also an important factor that needs to be regulated for long term real time cell culture studies to be viable.

CHAPTER 7

CONCLUSION AND FUTURE WORK

The overall aim of the project was to pattern the cell growth on the biodegradable polymer. We have successfully shown the patterning of polymer with different structures. Also we have got promising results with the fibroblast cells study in guiding of cell and cell alignment along the channel. The next aim is to pattern endothelial cells and also completion of 3D scaffold formation for vascularization.

The real time study presents, a new microfluidics system capable of culturing cells using microfluidic technology. This microfluidic system was fabricated using MEMS techniques. We are successfully able to grow cells outside incubator and cell movement was also observed. Seeking better real time cells' behavioral data, we are investigating control parameters such as CO_2 and pH control.

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